

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
1 March 2001 (01.03.2001)

PCT

(10) International Publication Number  
**WO 01/13943 A3**

- (51) International Patent Classification<sup>7</sup>: A61K 39/00, 39/02, 39/002, 39/385, A61P 31/04, 33/02
- (21) International Application Number: PCT/GB00/03225
- (22) International Filing Date: 18 August 2000 (18.08.2000)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:  
9919733.7 19 August 1999 (19.08.1999) GB
- (71) Applicant (for all designated States except US): **IMMUNOBIOLOGY LIMITED** [GB/GB]; Babraham Biocubators, Babraham, Cambridge CB2 4AT (GB).
- (72) Inventor; and
- (75) Inventor/Applicant (for US only): **COLACO, Camilo, Anthony, Leo, Selwyn** [GB/GB]; 107 Foster Road, Cambridge CB2 2JN (GB).
- (74) Agents: **DUMMETT, Thomas, Ian, Peter** et al.; Dummett Copp, 25 The Square, Martlesham Heath, Ipswich IP5 3SL (GB).
- (81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.
- (84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).
- Published:**  
— with international search report
- (88) Date of publication of the international search report:  
20 September 2001
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*



**WO 01/13943 A3**

(54) Title: STRESS PROTEIN-PEPTIDE COMPLEXES AS VACCINES AGAINST INTRA CELLULAR PATHOGENS

(57) Abstract: The present invention relates to a method for producing and isolating specific immunogenic heat shock proteins induced by heat or tumour necrosis factor treatment of cells infected by intra-cellular pathogens; and vaccines prepared from such proteins.



# INTERNATIONAL SEARCH REPORT

Int'l Application No  
PCT/GB 00/03225

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 7 A61K39/00 A61K39/02 A61K39/002 A61K39/385 A61P31/04  
A61P33/02

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

CANCERLIT, EMBASE, CHEM ABS Data, SCISEARCH, MEDLINE, BIOSIS, EPO-Internal

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 34641 A (UNIV FORDHAM) 13 August 1998 (1998-08-13) page 24, line 15 -page 25, line 32	9-15
A	---	1-8
X	HEIKEMA A ET AL: "Generation of heat shock protein-based vaccines by intracellular loading of gp96 with antigenic peptides." IMMUNOLOGY LETTERS, (1997 JUN 1) 57 (1-3) 69-74. XP000982318 the whole document	9-15
	---	
	-/--	

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

\*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

- \*X\* document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

\*G\* document member of the same patent family

Date of the actual completion of the international search

19 February 2001

Date of mailing of the international search report

01/03/2001

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl  
Fax. (+31-70) 340-3016

Authorized officer

Covone, M



# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/GB 00/03225

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9834641 A	13-08-1998	US 6017540 A AU 724772 B AU 6145598 A EP 0973548 A ZA 9800978 A	25-01-2000 28-09-2000 26-08-1998 26-01-2000 17-08-1998
WO 0010597 A	02-03-2000	AU 5429799 A	14-03-2000



## PATENT COOPERATION TREATY

## PCT

## INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference <b>P2661PC/TIPD</b>	<b>FOR FURTHER ACTION</b> See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)	
International application No. <b>PCT/GB00/03225</b>	International filing date (day/month/year) <b>18/08/2000</b>	Priority date (day/month/year) <b>19/08/1999</b>
International Patent Classification (IPC) or national classification and IPC <b>A61K39/00</b>		
Applicant <b>IMMUNOBIOLOGY LIMITED et al</b>		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.



2. This REPORT consists of a total of 7 sheets, including this cover sheet.

- ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of 3 sheets.

3. This report contains indications relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☒ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☒ Certain observations on the international application

Date of submission of the demand <b>13/03/2001</b>	Date of completion of this report <b>26.11.2001</b>
Name and mailing address of the international preliminary examining authority:  European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer <b>Renggli, J</b> Telephone No. +49 89 2399 7461 





**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT**

International application No. PCT/GB00/03225

**I. Basis of the report**

1. With regard to the **elements** of the international application (*Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17)*):

**Description, pages:**

1-18 as originally filed

**Claims, No.:**

1-13 with telefax of 05/11/2001

**Drawings, sheets:**

1/3-3/3 as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims. Nos.:



**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT**

International application No. PCT/GB00/03225

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

*(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)*

6. Additional observations, if necessary:

**III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability**

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

- ☐ the entire international application.
- ☒ claims Nos. 12-13 with respect to industrial applicability.

because:

- ☒ the said international application, or the said claims Nos. 12-13 with respect to industrial applicability relate to the following subject matter which does not require an international preliminary examination (*specify*):  
**see separate sheet**
- ☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):
- ☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.
- ☐ no international search report has been established for the said claims Nos. .

2. A meaningful international preliminary examination cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

- ☐ the written form has not been furnished or does not comply with the standard.
- ☐ the computer readable form has not been furnished or does not comply with the standard.

**V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability;  
citations and explanations supporting such statement**

1. Statement

Novelty (N)

Yes: Claims 1-13



International application No. PCT/GB00/03225



**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT - SEPARATE SHEET**

International application No. PCT/GB00/03225

**ITEM III:**

Claims 12 and 13 relate to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Article 34(4)(a)(i) PCT).

**ITEM V:**

1. Reference is made to the following document:

D1 WO 98/34641

D2 Heikema A. et al., Immunology Letters, 1997, Vol. 57, pp. 69-74

2. Industrial applicability (Art. 33(4) PCT):

The subject-matter of claims 1-11 is susceptible of industrial application.

For the assessment of the present claims 12 and 13 on the question whether they are industrially applicable, no unified criteria exist in the PCT Contracting States. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

3. Novelty (Art. 33(2) PCT):

D1 and D2, which are considered to be the closest prior art documents, disclose methods for producing complexes comprising heat-shock proteins and antigenic peptides. Neither in D1, nor in D2 were the complexes obtained after heat treatment or after tumour-necrosis factor treatment. Indeed, the said complexes were obtained either from tumour cells maintained under standard culture conditions and then mixed with an infectious agent's antigen or from cells infected with a recombinant SFV virus (see D1, page 11; pages 24-25 and D2, page 71, chapter 2.7).





**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT - SEPARATE SHEET**

International application No. PCT/GB00/03225

The subject-matter of claims 1-7 is thus considered to be novel over the cited prior art.

As it has been shown in the present application that stress-induced products obtained from *M. tuberculosis* or *P. berghei* infected cells gave a weaker immune response than stress-induced products obtained from corresponding infected cells stressed by heat or tumour-necrosis factor (see examples 4 and 5 of the present application, pages 15-18) novelty can also be acknowledged for the subject-matter of claims 8-11 and 12-13 of the present application.

4. Inventive step (Art. 33(3) PCT):

The problem to be solved by claim 8 over D1 and D2 can be seen as the provision of a more immunogenic vaccine comprising one ore more complexes between a shock protein and an antigenic fragment.

The solution consists in treating a cell infected with a bacterial, protozoal or parasitic intra-cellular pathogen with heat or with tumour necrosis factor.

This solution is not suggested in the cited prior art and the subject-matter of claims 8-11 and 12-13 is thus considered to be inventive.

The same remark consequently applies for the methods of claims 1-7.

**ITEM VI:****Certain published documents (Rule 70.10)**

Application No Patent No	Publication date (day/month/year)	Filing date (day/month/year)	Priority date (valid claim) (day/month/year)
WO 00/10597	02.03.2000	19.08.1999	19.08.1998

The above document (from the applicant), which has an earlier priority date than the present application may become relevant (i) in the regional phase of the application and (ii) may be relevant in the examination of novelty and inventive step for those parts of the application, if any, which do not have a valid claim to priority.



**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT - SEPARATE SHEET**

International application No. PCT/GB00/03225

This document pertains to a method for producing a vaccine, comprising the treatment with a cytokine like tumour necrosis factor alpha of a virally infected cells, the extraction of the heat shock proteins from the cells and the use of the extracted proteins in the preparation of a vaccine.

**ITEM VIII:**

Claim 7 would appear to be superfluous. It is not clear how the application of stress in claim 1 could be carried out in vivo; moreover, this has not been shown in the present application which is apparently limited to in vitro methods.



1.1-2001

GB0003225

19

1 Claims

2

3 1. A method for producing a vaccine containing an  
4 immunogenic determinant, characterised in that it  
5 comprises the steps of:

6 a) subjecting cells infected with an intra-  
7 cellular bacterial, protozoan or parasitic pathogen  
8 to stress with heat or tumour necrosis factor; and

9 b) extracting the endogenous stress-induced  
10 products from the stressed cells; and

11 c) using the extracted products as the immunogenic  
12 determinant in the preparation of the vaccine  
13 composition.

14

15 2. A method as claimed in claim 1, characterised  
16 in that the active ingredient of the immunogenic  
17 determinant consists predominantly of one or more  
18 shock protein/antigenic peptide fragment complexes.

19

20 3. A method as claimed in either of claims 1 or 2,  
21 characterised in that the cells are infected by  
22 bacterial pathogens and the stress applied is heat.

23

24 4. A method as claimed in claim 3, characterised  
25 in that the heat stress is achieved by heating to  
26 from 5 to 8° above the normal temperature of  
27 cultivation of the cells.

28

29 5. A method as claimed in claim 1, characterised  
30 in that the cells are infected by parasitic  
31 pathogens and the stress is induced by tumour  
32 necrosis factor.

AMENDED SHEET

Empfandscell 3 NOV 1990



11-2001

MURG TROYO AND CO

3078401

GB0003225

20

1 6. A method as claimed in any one of the preceding  
2 claims, characterised in that the cells have been  
3 modified to induce synthesis of stress proteins.

4  
5 7. A method as claimed in any of the preceding  
6 claims, characterised in that the application of  
7 stress to the cells is carried out in vitro.

8  
9 8. A vaccine composition containing an immunogenic  
10 determinant, characterised in that the immunogenic  
11 determinant comprises one or more complexes between  
12 a shock protein and an antigenic peptide fragment  
13 derived from the heat or tumour necrosis factor  
14 stressing of a cell infected with a bacterial,  
15 protozoal or parasitic intra-cellular pathogen.

16  
17 9. A vaccine composition containing an immunogenic  
18 determinant, characterised in that the immunogenic  
19 determinant is produced by a method as claimed in  
20 any one of claims 1 to 7.

21  
22 10. A vaccine composition as claimed in either of  
23 claims 8 or 9, characterised in that the composition  
24 also contains an adjuvant for the immunogenic  
25 determinant.

26  
27 11. A vaccine composition as claimed in any one of  
28 claims 8 to 10, characterised in that the  
29 composition is an aqueous composition.

30  
31 12. A method for treating an animal with a vaccine  
32 characterised in that it comprises administering a

AMENDED SHEET

Enofanastat 5 NOV 1988





11-2001

GB0003225

21

1 pharmaceutically acceptable quantity of a vaccine  
2 composition as claimed in any one of claims 8 to 11  
3 sufficient to elicit an immune response in the  
4 animal.

5

6 13. A method as claimed in claim 12, characterised  
7 in that the vaccine composition is administered by  
8 injection.



## PATENT COOPERATION TREATY

## PCT

## INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference <b>P2661PC/TIPD</b>	<b>FOR FURTHER ACTION</b> see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
International application No. <b>PCT/GB 00/03225</b>	International filing date (day-month-year) <b>18/08/2000</b>	(Earliest) Priority Date (day-month-year) <b>19/08/1999</b>
Applicant  <b>IMMUNOBIOLOGY LIMITED</b>		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 5 sheets.

☒ It is also accompanied by a copy of each prior art document cited in this report.

## 1. Basis of the report

- a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

☐ the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

- b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing:

☐ contained in the international application in written form.

☐ filed together with the international application in computer readable form.

☐ furnished subsequently to this Authority in written form.

☐ furnished subsequently to this Authority in computer readable form.

☐ the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

☐ the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. ☒ **Certain claims were found unsearchable** (See Box I).

3. ☐ **Unity of invention is lacking** (see Box II).

4. With regard to the **title**,

☐ the text is approved as submitted by the applicant.

☒ the text has been established by this Authority to read as follows:

**STRESS PROTEIN-PEPTIDE COMPLEXES AS VACCINES AGAINST INTRA CELLULAR PATHOGENS**

5. With regard to the **abstract**,

☒ the text is approved as submitted by the applicant.

☐ the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No.

☐ as suggested by the applicant.

☐ because the applicant failed to suggest a figure.

☐ because this figure better characterizes the invention.

☒

None of the figures.



## INTERNATIONAL SEARCH REPORT

International Application No.

/GB 00/03225

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A61K39/00 A61K39/02 A61K39/002 A61K39/385 A61P31/04  
A61P33/02

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

CANCERLIT, EMBASE, CHEM ABS Data, SCISEARCH, MEDLINE, BIOSIS, EPO-Internal

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
X	WO 98 34641 A (UNIV FORDHAM) 13 August 1998 (1998-08-13) page 24, line 15 -page 25, line 32	9-15
A	---	1-8
X	HEIKEMA A ET AL: "Generation of heat shock protein-based vaccines by intracellular loading of gp96 with antigenic peptides." IMMUNOLOGY LETTERS, (1997 JUN 1) 57 (1-3) 69-74. , XP000982318 the whole document ---	9-15
	--- -/--	

☒ Further documents are listed in the continuation of box C☒ Patent family members are listed in annex

## Special categories of cited documents

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*I\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- \*Z\* document member of the same patent family

Date of the actual completion of the international search

19 February 2001

Date of mailing of the international search report

01/03/2001

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo.nl  
Fax (+31-70) 340-3016

Authorized officer

Covone, M



## INTERNATIONAL SEARCH REPORT

International Application No

/GB 00/03225

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document with indication where appropriate, of the relevant passages	Relevant to claim No
A	MULTHOFF G: "HEAT SHOCK PROTEIN 72 (HSP72). A HYPERTHERMIA-INDUCIBLE IMMUNOGENIC DETERMINANT ON LEUKEMIC K562 AND EWING'S SARCOMA CELLS." INTERNATIONAL JOURNAL OF HYPERTHERMIA, vol. 13, no. 1, 1997, pages 39-48, XP000982278 ISSN: 0265-6736 the whole document ---	1-15
P, X	WO 00 10597 A (COLACO SUSANNA MARY ;IMMUNOBIOLOGY LTD (GB); COLACO CAMILO ANTHONY) 2 March 2000 (2000-03-02) example 2 claims -----	1,2,5-15





# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

EP/GB 00/03225

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 9834641	A	13-08-1998	US 6017540 A	25-01-2000
			AU 724772 B	28-09-2000
			AU 6145598 A	26-08-1998
			EP 0973548 A	26-01-2000
			ZA 9800978 A	17-08-1998
WO 0010597	A	02-03-2000	AU 5429799 A	14-03-2000



**PCT**WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification</b> <sup>7</sup> : <b>A61K 39/00, 38/00, C07K 14/47, 1/00</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 00/10597</b> <b>(43) International Publication Date:</b> 2 March 2000 (02.03.00)
<b>(21) International Application Number:</b> PCT/GB99/02607 <b>(22) International Filing Date:</b> 19 August 1999 (19.08.99)  <b>(30) Priority Data:</b> 9818133.2 19 August 1998 (19.08.98) GB  <b>(71) Applicant</b> ( <i>for all designated States except US</i> ): IMMUNOBI- OLOGY LIMITED [GB/GB]; 170 Foster Road, Cambridge CB2 2JN (GB).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants</b> ( <i>for US only</i> ): COLACO, Camilo, An- thony, Leo, Selwyn [GB/GB]; (GB). COLACO, Susanna, Mary [GB/GB]; 170 Foster Road, Cambridge CB2 2JN (GB).  <b>(74) Agents:</b> GEMMELL, Peter, Alan et al.; Dummett Copp, 25 The Square, Martlesham Heath, Ipswich IP5 3SL (GB).		<b>(81) Designated States:</b> AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the</i> <i>claims and to be republished in the event of the receipt of</i> <i>amendments.</i>
<b>(54) Title:</b> VACCINE COMPRISING CYTOKINE-INDUCED HEAT SHOCK PROTEINS (HSP)		
<b>(57) Abstract</b>  The present invention relates to a method for producing and isolating specific immunogenic heat shock proteins from cancer cells or virally infected or recombinant cells and vaccines prepared from such proteins.		

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakhstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

## VACCINE COMPRISING CYTOKINE-INDUCED HEAT SHOCK PROTEINS (HSP)

The present invention relates to a vaccine and a method for producing a vaccine.

5 BACKGROUND OF THE INVENTION

An important component of any human immune response is the presentation of antigens to T cells by antigen presenting cells (APCs) such as macrophages, B cells or dendritic cells. Fragments of foreign antigens are presented on the surface of the macrophage in  
10 combination with Major histocompatibility complex (MHC) molecules, in association with helper molecules, such as CD4 and CD8 molecules. Such "antigenic fragments", presented in this way, are recognised by the T cell receptor of T cells, and the interaction of the antigen with the T cell receptor results in antigen-specific T cell proliferation, and secretion of lymphokines by the T-cells.

15 The nature of the antigenic fragment presented by the APCs is critical in establishing immunity. Heat shock proteins (HSPs) form a family of highly conserved proteins that are widely distributed throughout the plant and animal kingdoms. On the basis of their molecular weights, HSPs are grouped into six different families: small (hsp 20-30kDa);  
20 hsp40; hsp60; hsp70; hsp90; and hsp100. Although HSPs were originally identified in cells subjected to heat stress, they have been found to be associated with many other forms of stress such as infections, and are thus more commonly known as "stress proteins" (SPs).

Members of the mammalian hsp90 family include cytosolic hsp90 (hsp83) and the  
25 endoplasmic reticulum counterparts hsp90 (hsp83), hsp87, Grp94 (Erp99) and gp97. See for instance, Gething et al. (1992) *Nature* 355:33-45. Members of the hsp70 family include cytosolic hsp70 (p73) and hsp70 (p72), the endoplasmic reticulum counterpart BiP (Grp78), and the mitochondrial counterpart hsp70 (Grp75). Members of the mammalian hsp60 family have only been identified in the mitochondria

30 SPs are ubiquitous within cells, one of the roles of SPs is to chaperone peptides from one cellular compartment to another and to present peptides to the MHC molecules for cell surface presentation to the immune system. In the case of diseased cells, SPs also chaperone

viral or tumour-associated peptides to the cell-surface. Li and Srivastava (1994) *Behring Inst. Mitt.* 94: 37-47 and Suzue et al. (1997) *Proc.Natl.Acad.Sci. USA* 94: 13146-51. The chaperone function is accomplished through the formation of complexes between SPs and proteins and between SPs and viral or tumour-associated peptides in an ATP-dependent reaction. SPs bind a wide spectrum of peptides in an ATP dependent manner. The bound peptides appear to be a random mix of peptides. The mixtures and exact natures of the peptides have not been determined. The association of SPs with various peptides has been observed in normal tissues as well and is not a tumour-specific phenomenon. See Srivastava (1994) *Experimentia* 50: 1054-60.

For instance, expression of hsp26, hsp60, hsp70 and hsp90 on the surface of human chronic myeloid leukemia (CML) cells from patients has been observed, Chant et al. (1995) *Br.J.Haematol.* 90: 163-8. Cell surface expression of hsp70 has been detected on normal, pre-malignant and malignant human oral mucosa, Kaur et al. (1998) *Oral Oncol.* 34: 93-8. A correlation of hsp70 expression with clinico-pathological features showed a positive association with the severity of dysplasia in oral mucosal epithelium.

Ito et al. (1998) *J.Oral.Pathol.Med.* 27: 18-22, reported that they examined 24 specimens of squamous cell carcinoma of the tongue and found that, although SP immunohistochemistry revealed changes in expression during tumorigenesis, there was no observed correlation with other clinical features studied (survival period, stage, lymph node metastasis, histological grade or p53 immuno-staining)

It is currently believed that the antigenicity of SPs results not from the SP per se, but from the complex of peptide associated with the SP. This conclusion is based on a number of characteristics of the complexes. There are no differences in the structure of SPs derived from normal and tumour cells. Certain complexes lose their immunogenicity upon treatment with ATP, Udonio et al. (1993) *J.Exp.Med.* 178: 1391-96. Such loss of immunogenicity is due to dissociation of the complex into its SP and peptide components.

In a therapeutic context, it has been proposed to use SP-antigen complexes as vaccines. In particular, US Patent No. 5,750,119 to Srivastava discloses a multi-step, cancer patient-specific method for inhibiting the proliferation of a tumour in a mammal, by (a) removing

tumour from the mammal; (b) isolating all complexes from the tumour cells and (c) administering the isolated complexes back to the mammal in order to stimulate a tumour-specific immune response. Hsp70-peptide, hsp90-peptide and gp96-peptide complexes are itemised as complexes having particular vaccine utility. Nevertheless, in the practice of the method disclosed by Srivastava, it is not considered necessary or even practical to isolate a specific peptide involved or even the particular complex in eliciting the immune response.

WO 97/10000 and WO 97/10001 disclose that a mixture of heat shock proteins (HSPs) isolated from cancer cells or virally infected cells are capable eliciting protective immunity or cytotoxic T lymphocytes to the cognate tumour or viral antigen. However, in contrast, HSPs isolated from normal cells are unable to elicit such immunity. It is now thought that HSPs are not immunogenic *per se*, but are able to elicit immunity because of their association with tumour or virus specific antigenic peptides that are generated during antigen processing. Specifically, the peptides associated with the HSPs are immunogenic, and are presented to the T cells. HSPs stripped of associated peptides lose their immunogenicity (Udono, H. and Srivastava, P. K., Journal of Experimental Medicine, 178, page 1391 *ff*, 1993). To date, the nature of these peptides has not been determined.

The immunogenicity of HSP preparations depends upon the presence of phagocytic cells, such as macrophages and other APCs. It is now thought that HSPs are taken up by macrophages, and those peptides associated with the HSPs are then presented by MHC class I molecules of the macrophage. In this way, a T cell response is initiated.

The use of HSP proteins as vaccine components has been disclosed in WO 95/24923 and WO 98/34641. HSPs isolated from tumour cells or viral infected cells have been suggested as a source of antigenic peptides, which could then be presented to T cells. This necessitates the production and purification of HSPs from such cells. In order to help the purification, cells may be treated by heat shock or other stresses, to increase intracellular levels of the SPs. However, it is not presently known if such induced SPs will form immunogenic complexes with heterologous peptides as do constitutively expressed HSPs.

The stimulation of cells by heat shock produces a general increase in the level of heat shock proteins. Ideally, it would be desirable to stimulate the production of only a subset of HSPs,

which are especially immunogenic. Such a subset of HSPs would be particularly suitable for use in the production of a vaccine. A number of other stresses such as osmotic stress, oxidative stress or heavy-metal treatment are known to produce closely related HSPs. However, at present, there is no way to specifically stimulate cells to produce such a subset of HSPs with enhanced immunogenicity.

### SUMMARY OF THE INVENTION

Therefore, in a first aspect, the present invention provides a method for producing a vaccine, comprising the steps of:

- a) treating virally infected, recombinant or cancerous cells with a cytokine;
- b) extraction of heat shock proteins from the treated cells; and
- c) use of the extracted heat shock proteins in the preparation of a vaccine.

It is surprising that the treatment of virally infected, transformed or cancerous cells with cytokines, particularly interferon- $\alpha$ , produces HSPs which are more immunogenic than HSPs derived from cells which have been heat shocked. The most notable aspect of immunity elicited by cytokine-induced HSPs is the long-term memory compared to that induced by immunisation by other HSP subsets.

The term "vaccine" as used herein, refers to any composition which stimulates the immune system such that it can better respond to subsequent infections. It will be appreciated that a vaccine usually contains an immunogenic determinant and an adjuvant, which non-specifically enhances the response to that determinant.

Preferably, the immunogenic determinant for the present invention is delivered in combination with an adjuvant. Suitable adjuvants are readily apparent to the person skilled in the art, such as Freund's complete adjuvant, Freund's incomplete adjuvant, Quil A, Detox, ISCOMs or squalene. However, it will be appreciated that the vaccine of the present invention may also be effective without an adjuvant. Such a vaccine may be given by any suitable means, such as orally, or by injection.



The term "heat shock protein", as used herein, is standard in the art, and includes those proteins which are induced when a cell is subjected to heat stress, such as HSP70 and GRP96. These proteins play a role in the protection of other cellular proteins from the effects of heat stress. Heat shock proteins (HSPs) are also expressed under normal cellular conditions, where they play a role in protein folding. The family of HSPs includes stress proteins (SPs) and the members of this family of proteins that are both constitutively expressed as well as those induced by a variety of other stresses including environmental insults and oxidative and osmotic stresses. The family of HSPs is defined primarily on amino acid sequence homologies.

Eucaryotic cells contain a number of families of HSPs, such as the HSP90 family, the HSP70 family, the HSP60 family and the GRP96 family (endoplasmic reticulum chaperonin). These families are named on the basis of the size of the peptides which they encode. The families are highly conserved between species. In addition, many bacteria also express homologues of eucaryotic proteins. Preferably the vaccine contains at least one HSP derived from the cytoplasm of the virally infected, recombinant or cancerous cell, and one that is membrane bound. We particularly prefer that the HSP70, HSP90 and the GRP96 families of proteins are used as immunogenic determinants in the present invention, with HSP70 and GRP96 most preferred. Preferably the immunogenic proteins have greater than 25% homology and/or 20% identity at the amino acid level to the heat-induced HSP protein families.

The term "cytokine", as used herein, is standard in the art, and refers to an intercellular chemical signal. The present invention requires that the cytokine that is used is able to stimulate the presence of HSPs within virally infected or tumour cells. We prefer that the cytokine is an interferon, especially interferon- $\alpha$  (IFN- $\alpha$ ). However, it will be appreciated that any suitable cytokine which is able to specifically induce the upregulation of a subset of immunogenic HSPs is included in the present invention. These cytokines include, but are not limited to, interferon- $\beta$  (IFN- $\beta$ ), tumour necrosis factor  $\alpha$  (TNF $\alpha$ ), interleukins 1 and 6 (IL1 and IL6).

Treatment of virally infected, recombinant or cancer cells with IFN- $\alpha$  elicits HSPs that are more immunogenic, by weight, than those HSPs derived from cells that have been heat

shocked, producing higher titres of antibodies and/or frequencies of cytotoxic T-cells. It will be appreciated that these HSPs carry the antigenic peptides derived from the cancer cell or carry the fragments of the viral peptides. Without being constrained by theory, it is thought that cytokine treatment works either to specifically induce those HSPs most able to interact with peptides, or to induce those HSPs which are most easily phagocytosed by APCs, or both. The cytokine may also act to increase levels of viral or tumour peptides, which are then able to interact with the HSPs and, thus, are suitable for effective stimulation of the immune system.

10 In the case of treatment with other cytokines, the present invention includes any suitable method of cytokine treatment which produces a subset of HSPs that are more immunogenic, by weight, than those produced by heat shock alone. Comparative immunogenicity can be determined by *in vivo* testing on animal models. Other suitable methods will be readily apparent to the person skilled in the art, 'Current Protocols in Immunology', Wiley Interscience, 1997.

The tumour-derived, transformed or virally infected cells are preferably treated with the cytokine *in vitro*. Cytokines are suitably used at 0.5-1000 international units (i.u)/ml of media, preferably about 1-500 i.u/ml. Specifically, for IFN- $\alpha$  we prefer that cells are treated with 10-500 i.u./ml and are then cultured for 10-16 hours. Alternatively, cells may be grown on cytokine-producing feeder-monolayers or induced to produce endogenous cytokines. However, it will be appreciated that some cells are more sensitive than other, and thus a greater or lesser amount of cytokine may be needed. For example, IFN- $\alpha$  is effective at 0.1-2 pg per ml of media with an ECV infected fibroblast cell line 2D9 but at 1-100 pg with the B6 cell line Daudi. Moreover, the incubation time of cells with cytokines is also variable. We prefer that a 6-18 hour exposure time is used, but this time may be reduced to 0.5-4 hours in some cases and still be effective.

30 The means to test for optimum cytokine levels and incubation period are readily available to the person skilled in the art. However, it will be appreciated that the exact level of cytokine is not crucial to the invention, as long as the treatment stimulates the production of the desired immunogenic HSPs within the treated cells. Similarly, the other conditions of treatment, such as the length of exposure to cytokine, cell incubation media and temperature

of incubation are not essential features of the present invention and may be varied depending upon the exact nature of the cell population and cytokine that is used. Means to vary and optimise these parameters will be readily apparent to the person skilled in the art.

- 5 Preferably the cytokine is isolated from the same organism as the cell which is to be treated. Treatment of cells with cytokines from the same organism provides optimum stimulation of HSPs. However, the use of cytokines from other species or individuals may also be useful in up-regulation of the HSP levels in cells, to provide suitable HSPs for use in the present invention.

10

- The terms 'virally infected', 'recombinant' and 'tumour' cells are standard in the art. Cells suitable for use in the present invention include any form of tumour cell, cells infected with any virus and cells transformed to express heterologous proteins or peptides. Such cells contain antigens which are not usually present in uninfected or normal cells or cell lines, or  
15 which are not normally presented to the immune system. Fragments of such peptides which interact with HSPs are able to stimulate the body's immune response to such abnormal cells. In the case of recombinant cell lines, the heterologous antigens expressed include any molecules to which an immune response is desired, including protozoan, parasitic, fungal and bacterial antigens.

20

- Any suitable virally infected, recombinant or tumour cell can be used in the present invention, to provide a source of HSP. In the case of tumour cells, we prefer that the cells used for extraction of HSPs are cells derived directly from the tumour cells, although transformed cells are also appropriate. Methods for the transformation of cells extracted  
25 from tumours, for example, are well known and standard in the art. Furthermore, means for isolating tumorigenic or virally infected cells are well known, and may involve such techniques as biopsies, surgery, use of blood cells or cell scrapings from the throat or mouth. Recombinant cells can be produced by transfection, retroviral infection and other methods well known in the art. Other methods for the isolation of tumorigenic or virally  
30 infected cells will be readily apparent to the person skilled in the art.

In addition, we prefer that virally infected cells are obtained by infection of an appropriate cell line with the desired virus *in vitro*. Cells infected in this way can then be stimulated to

produce antigenic HSPs, suitable for vaccination against that virus. This includes recombinant viruses that carry antigenic epitopes from a heterologous source such as those used to produce recombinant vaccines. These also include all types of transfected recombinant cells used to produce recombinant vaccines, such as mammalian cell lines  
5 transfected with recombinant vectors by standard methods in the art such as electroporation, liposome fusion and calcium phosphate. Furthermore, the invention also includes the use of HSPs from eucaryotic cells that respond to treatment of cytokines, including those expressing homologous, and recombinant cell lines expressing heterologous antigens. While the antigens are predominantly proteins and peptides, they can also include  
10 carbohydrate, nucleic acid and lipid moieties that bind HSPs.

It will be appreciated that HSPs derived from tumour cells, for example, will in general only be able to provide immune protection against the specific tumour from which the cells were derived. In order to provide the best vaccine protection, it would therefore be desirable to  
15 combine HSP preparations derived from a number of different tumours. This could provide immune protection against the full range of tumours, by exposing the immune system to antigens from each different type of tumour cell. Alternatively multiple tumour antigens, such as melanoma-associated antigens (MAGEs) and prostate specific antigen (PSA), can be expressed in recombinant cells which are then used to produce the cytokine induced  
20 HSPs. Similar arguments apply equally to antigens derived from virally infected cells. In the case that HSPs from different tumours are combined to form a vaccine, then we prefer that the HSP preparations are derived from the same species as the organism which is to be vaccinated. In the case of recombinant antigens and viral antigens heterologous HSP vaccines are equally preferred.

25 Moreover, it will be appreciated that a patient-specific cancer vaccine is also encompassed by the invention. The vaccine can be prepared by isolating a specific patient's tumour cells, for example, and treating the cells with a cytokine to stimulate the production of HSPs. The HSPs produced in this way could then be purified and be used in a vaccine, to stimulate the  
30 patient's specific immune response to his or her own tumour.

It will further be appreciated that different tumours may share the same antigenic peptides, in which case a HSP preparation derived from one specific tumour line would provide

immune protection against other tumours, which also share the same antigenic peptide fragment. Recombinant cells expressing tumour antigens can also be used to produce effective HSP vaccine preparations.

- 5 The extraction and purification of HSP proteins from virally infected cells or tumour cells is standard in the art. Suitable methods include disruption of treated cells by homogenisation or sonication, followed by centrifugation to obtain a crude HSP preparation in the supernatant. In addition, ConA fractionation or ADP binding columns can also be used to purify the HSPs. Other suitable methods for the extraction of HSPs are readily available to  
10 the person skilled in the art, see for example those described in WO 97/10000 and WO 97/10001.

It will be appreciated that the present invention also includes methods of producing and isolating specific immunogenic HSPs from cancer cells, recombinant cells or virally infected  
15 cells, essentially as described above. HSPs derived in this way may be used to isolate samples of antigenic peptides, for example.

Methods of medical treatment using the vaccine of the present invention are also included in the present invention. Such methods include the administration of a pharmaceutically  
20 acceptable quantity of immunogenic determinant, optionally in combination with an adjuvant, sufficient to elicit an immune response in a patient.

### Examples

The following examples are provided to illustrate, but not limit, the described invention.

25

#### **Example 1**

##### *Preparation of cytokine-induced SPs.*

One specific example of the HSP preparation process is as follows. Cancerous or virally infected cells are incubated in a serum free media, such as RPMI (Sigma), with a suitable  
30 cytokine overnight. Cells are then washed in serum-free media, followed by a wash in phosphate buffered saline (PBS). The cells are then re-suspended in homogenisation buffer. The homogenisation buffer may be a hypotonic buffer, such as 10 mM phosphate pH 7.4 with 2mM MgCl<sub>2</sub>, after which the cells are then disrupted using a cell homogeniser (e.g. a

Dounce or Potter homogeniser, Ultraturrax or Waring blender). Alternatively, the homogenisation buffer may contain detergent, such as PBS with 0.5% Tween, the detergent concentration being between 0.1-1% and suitable to solubilise the cell membrane. The cell lysate is then treated by centrifugation, typically 3-5000 x g for 5 minutes, to remove the nuclei and cell debris, followed by a high speed centrifugation step, typically 100,000g for 15-30 minutes.

The supernatant thus obtained is processed to give a protein complex suitable for use in a vaccine. This can be done simply by ammonium sulphate precipitation which uses a 50-70% ammonium sulphate cut. Specifically, 50 % (w/w) ammonium sulphate is added at 4°C, the precipitate is discarded, followed by the addition of more ammonium sulphate to bring the concentration to 70 %. The protein precipitate is harvested by centrifugation, and then dialysed into an appropriate physiological, injectable buffer, such as saline, to remove the ammonium sulphate before use. It will be appreciated that the HSPs isolated in this way are not purified to homogeneity, but are nevertheless suitable for use as a vaccine component.

If more purified HSP preparation is required, then the HSPs may be purified from the supernatant by affinity chromatography on matrices carrying adenosine diphosphate, such as ADP-agarose or ADP-sepharose. These methods are standard in the art, and are outlined in WO 97/10000, WO 97/10001 and WO 97/10002.

HSPs may be used at any suitable concentration. We prefer that the amount of HSP70 and HSP90 complex that is administered is in the range of 10-600 µg, preferably 10-100 µg, most preferably 25 µg. For HSP90 complexes we prefer that levels used are 50-5000 µg, preferably 100 µg.

In order to determine the immunogenicity of stress protein-peptide complexes, T cell proliferation assays may be used. Suitable assays include the mixed-lymphocyte reaction (MLR), assayed by tritiated thymidine uptake, and cytotoxicity assays to determine the release of <sup>51</sup>Cr from target cells. Both of these assays are standard in the art (see 'Current Protocols in Immunology', Wiley Interscience, 1997). Alternatively, antibody production

may be examined, using standard immunoassays or plaque-lysis assays, or assessed by interuterine protection of a foetus. (see 'Current Protocols in Immunology', *supra*).

#### Example 2

5 *Immunisation with cytokine induced HSPs; immunity in vaccine recipient*

Mouse 3T3 cells infected with VSV or recombinant 3T3 cells expressing with VSV G-protein were treated overnight with 5pg/ml interferon- $\alpha$ . HSPs were prepared from detergent lysates (0.25% Triton) by fractionation of the cellular extracts to produce a 50-70% ammonium sulphate fraction. Mice and rabbits were vaccinated with 1-10 micrograms  
10 of the stress-protein containing extract in phosphate buffered saline and boosted with identical vaccine dosages a month after the primary injection. Induction of immunity to VSV was assayed using an ELISA to detect antibody against VSV G-protein. Antibody titres of 1:1-10,000 were routinely obtained. Cytotoxic T-cell activity directed against VSV G-protein could also be detected in the HSP-immunised mice. Challenge of the rabbits with  
15 fixed VSV particles at 6, 12 and 18 months periods after the initial immunisations resulted in the production of good antibody responses with titres of 1:1-10 000 indicating good memory responses in the immunised animals.

#### Example 3

20 *Immunisation with cytokine induced HSPs; protection against live challenge.*

Mouse 3T3 cells expressing Sheep pestivirus E1/2 and NS3 proteins were treated overnight with interferon- $\alpha$  and HSPs were prepared from detergent lysates as described in Example 1. Sheep were vaccinated with 10-50 micrograms of HSPs in phosphate buffered saline and boosted with identical vaccine dosages a month after the primary injection. Production of  
25 antibodies against E1/2 proteins was detected by Western blotting. Challenge of animals with live virus ( $3 \times 10^6$ ) showed maximum antibody responses at 1-2 weeks compared to 4-5 weeks in the controls and initial studies showed prevention of transmission of virus to the foetus in pregnant ewes. Most interestingly, the high antibody titres of  $>1$  in 100 000 appeared to completely inhibit viral replication in immunised animals as assayed by the  
30 detection of processed E1/2 antigens or core viral antigens in the serum of immunised animals.

**Claims**

- 1 A method for producing a vaccine, comprising the steps of:
- 5 a) treating virally infected, recombinant or cancerous cells with a cytokine in an amount sufficient to induce the production of heat shock related stress proteins;
- b) extracting the cytokine-induced heat shock proteins from the treated cells; and
- c) using the extracted heat shock proteins in the preparation of a vaccine.
- 10 2 A method as claimed in claim 1 wherein the cellular extract consists essentially of heat shock-related stress proteins.
- 3 A method as claimed in either of claims 1 or 2, wherein the cytokine is selected from interferons, tumour necrosis factor and interleukins.
- 15 4 A method as claimed in claim 3, wherein the cytokine is interferon  $\alpha$  or  $\beta$ .
- 5 A method as claimed in claim 3, wherein the cytokine is interleukin 1 or 6.
- 6 A method as claimed in claim 3, wherein the cytokine is tumour necrosis factor  $\alpha$ .
- 20 7. A method for producing a vaccine substantially as hereinbefore described in any one of the examples.
8. A vaccine obtained according to the method of either of claims 1 or 7.
- 25 9. A vaccine as claimed in claim 8, wherein the cellular extract consists essentially of heat shock related stress proteins.
10. A vaccine as claimed in claim 8, wherein the cytokine is selected from an interferon, tumour necrosis factor and interleukins.
- 30 11. A vaccine as claimed in claim 8, wherein the cytokine is interferon  $\alpha$  or  $\beta$ .



- 5

# INTERNATIONAL SEARCH REPORT

In [REDACTED] Application No  
PCT/GB 99/02607

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A61K 39/00 A61K 38/00 C07K 14/47 C07K 1/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
X	WO 97 10001 A (UNIV FORDHAM) 20 March 1997 (1997-03-20) cited in the application page 5, line 34 -page 7, line 4 page 9, line 29 -page 12, line 35 page 16, line 6 -page 19, line 32 page 23, line 20 -page 26, line 20 page 35, line 26 -page 38, line 29	8-13
Y	---	1-6
X	US 5 750 119 A (SRIVASTAVA PRAMOD K.) 12 May 1998 (1998-05-12) cited in the application column 3, line 18 -column 5, line 25; claims 1-48	8-13
Y	---	1-6
	--/--	

☒ Further documents are listed in the continuation of box C

☒ Patent family members are listed in annex

### Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- "Z" document member of the same patent family

Date of the actual completion of the international search

17 January 2000

Date of mailing of the international search report

11.02.00

Name and mailing address of the ISA

European Patent Office P B 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel: (+31-70) 340-2040, Tx 31 651 epo nl,  
Fax: (+31-70) 340-3016

Authorized officer

Muller-Thomalla, K

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/GB 99/02607

## C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication where appropriate of the relevant passages	Relevant to claim 1-6
X	WO 95 24923 A (SINAI SCHOOL MEDICINE ;SRIVASTAVA PRAMOD K (US); UDONO HEIICHIRO ( ) 21 September 1995 (1995-09-21) cited in the application page 9 line 4 -page 14, line 3 page 25, line 9 -page 39, line 25	8-13
Y	---	1-6
X	BLACHERE N E ET AL: "Heat shock protein vaccines against cancer" JOURNAL OF IMMUNOTHERAPY,US,RAVEN PRESS, NEW YORK, vol. 14, page 352-356 XP002037576 ISSN 1053-8550 summary: page 355, column 1, paragraph 2 -column 2, last paragraph	8-13
Y	---	1-6
X	METZ F. ET AL: "Interleukin-4 upregulates the heat shock protein Hsp90alpha and enhances transcription of a reporter gene coupled to a single heat shock element." FEBS LETTERS, (1996 APR 29) 385 (1-2) 25-8. XP002127261 page 25, column 2, paragraphs 1,2 page 27, column 2, paragraph 3 -page 28, column 1, paragraph 1	8-13
Y	---	1-6
X	WACHLIN, G. ET AL: "Cytokine - induced stress response in pancreatic islets from diabetes-prone BB and MHC -congenic BB.1A rats." EXPERIMENTAL AND CLINICAL ENDOCRINOLOGY & DIABETES, (1997) VOL. 105, NO. 4, PP. A46. MEETING INFO.: SATELLITE MEETING TO THE 16TH INTERNATIONAL DIABETES FEDERATION CONGRESS, JOINT MEETING WITH THE EUROPEAN ASSOCIATION FOR THE STUDY OF DIABETES ISLET, XP002127262 abstract	8-13
Y	-----	1-6

## INTERNATIONAL SEARCH REPORT

International application No  
PCT/GB 99/02607

### Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
  
Although claims 1-6 comprise a method of treatment step of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☒ Claims Nos.:  
because they relate to parts of the international Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:  
  
see FURTHER INFORMATION sheet PCT/ISA/210
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

### Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

#### Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest
- ☐ No protest accompanied the payment of additional search fees

## INTERNATIONAL SEARCH REPORT

International Application No. PCT/GB 99/02607

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 7,14 (whole) and 8(partly)-13(partly) (insofar in that they relate to claim 7)

The subject-matter of claims 7 and 14 has not been searched, as it is not defined in clear technical terms allowing a meaningful interpretation of the scope covered by the subject-matter of said claims.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

Application No

PCT/GB 99/02607

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9710001 A	20-03-1997	US 5637251 A	17-11-1998
		AU 703101 B	18-03-1999
		AU 7018196 A	01-04-1997
		EP 0859631 A	26-08-1998
US 5750119 A	12-05-1998	AU 709643 B	02-09-1999
		AU 2281995 A	26-04-1996
		CA 2201498 A	11-04-1996
		CN 1167440 A	10-12-1997
		EP 0784477 A	23-07-1997
		JP 10506628 T	30-06-1998
		WO 9610411 A	11-04-1996
WO 9524923 A	21-09-1995	US 5961979 A	05-10-1999
		AU 701732 B	04-02-1999
		AU 2100995 A	03-10-1995
		CA 2185651 A	21-09-1995
		EP 0750513 A	02-01-1997
		JP 10501520 T	10-02-1998

XP-000982278

INT. J. HYPERTHERMIA, 1997, VOL. 13, NO. 1, 39-48

P.D.	0000 1997	10
P.	39-48	

## Heat shock protein 72 (HSP72), a hyperthermia-inducible immunogenic determinant on leukemic K562 and Ewing's Sarcoma cells

G. MULTHOFF

Institut für Klinische Hämatologie, Marchioninistr. 25, 81377 Munich, FRG

(Received 29 April 1996; revised 25 July 1996; accepted 22 August 1996)

Following non-lethal heat stress (41.8°C) and a recovery period at 37°C, the inducible 72 kDa HSP (HSP72) is detectable selectively on the cell surface of human Ewing's Sarcoma (ES) and of leukemic K562 cells but not on EBV transformed B cells (B-LCL) which were generated from PBL of healthy human volunteers. The HSP72 expression was measured by flowcytometric analysis using a monoclonal antibody (moAb) that specifically recognizes HSP72, the inducible form of the HSP70 group. The major histocompatibility complex (MHC) class I expression, detected with the moAb W6/32 was not affected by non-lethal heat exposure and a recovery period at 37°C for 12 h: ES cells express MHC class I molecules on about 80% of the cells; K562 cells exhibited no MHC class I expression neither before nor after heat shock. Inhibition of RNA- (actinomycin D) or protein-synthesis (cycloheximide) prior to heat treatment completely inhibits the expression of HSP72 on the cell surface of both tumour cells, thus indicating that *de novo* protein synthesis is required for HSP72 cell surface expression. Since, apart from HSP72, protein synthesis in general is down-modulated by heat shock we speculate that HSP72 molecules that are expressed on the cell surface of tumour cells might be recruited from newly synthesized proteins. The heat-inducible HSP72 cell surface expression on tumour cells could be correlated with an increased sensitivity of leukemic and sarcoma cells to lysis mediated by NK effector cells. The results of cold target inhibition assays revealed that histologically different tumour cells (sarcoma and leukemic cells) that were exposed to non-lethal temperatures have to share a similar if not identical HSP72 immunogenic determinant.

**Key words:** Leukemic cells, sarcoma cells, HSP72 cell surface expression, hyperthermia, immune response.

### Abbreviations

AcD, actinomycin D; CHX, cycloheximide; FACScan, registered trademark of Becton Dickinson and Company for a fluorescence activated cell sorter; ES, Ewing's Sarcoma cells; HSP72, heat shock protein (*Mr* 72 kDa); kDa, kiloDalton; MHC, major histocompatibility complex; moAb, monoclonal antibody; PBL, peripheral blood lymphocytes; PBMC, peripheral blood mononuclear cells; PBS, phosphate buffered saline.

### 1. Introduction

Heat, cellular stress factors (e.g. virus transformation, hypoxia, arsenite) and cellular development and differentiation processes result in an increased synthesis of heat shock proteins (HSP) which are highly conserved proteins (Schlesinger 1982) with a wide phylogenetic representation (Bienz and Pelham 1987). On the basis of their *Mr* and sequence homology, HSP are grouped into different families (Lindquist 1986). The major interest within the last years has been drawn to study members of

the HSP70 group (Morimoto 1993) which are located in nearly every cellular compartment like the cytoplasm, Golgi, endoplasmic reticulum (ER), nuclei or nucleoli where they fulfill chaperoning functions (Pelham 1986, Chirico *et al.* 1988, Welch 1992). Members of the HSP70 group have been found to be involved in antigen processing and presentation (Vanbuskirk *et al.* 1989). Cell surface localization of HSP70 was documented for virus-infected cells (DiCesare *et al.* 1992), for cells derived from patients suffering from autoimmune diseases (Heufelder *et al.* 1992) and for tumour cells (Ullrich *et al.* 1986, Srivastava *et al.* 1986, Ferrarini *et al.* 1992, Li and Srivastava 1993). Recent data of our group are in line with these findings. A cell surface expression of HSP72, the major heat-inducible form of the HSP70 group, after non-lethal heat exposure (41.8°C) or cytostatic treatment (alkyl-lysophospholipid) was detectable selectively on different tumour cell types but not on normal cells by using immunological and biochemical methods (Multhoff *et al.* 1995a, Botzler *et al.* 1996). These observations are supported by data of Tamura (1993) and Sato (1991) who reported that the 70 kDa heat shock cognate protein is localized only on the cell surface of oncogene transformed rat fibrosarcoma cells but not on normal fibroblast cells. Furthermore, we correlated the stress-inducible, tumour specific cell surface expression of HSP72, with an increased sensitivity to lysis mediated by non-MHC restricted natural killer (NK) cells (Multhoff *et al.* 1995b, Botzler *et al.* 1996, Multhoff and Hightower 1996).

With respect to the tumour specific cell surface expression of HSP72 in the present study two major questions were addressed: (i) is *de novo* protein synthesis necessary for HSP72 cell surface expression; (ii) differ histologically different tumour cell types like sarcoma and leukemic cells in their immunogenic HSP72 determinant that is recognized by non-MHC restricted natural killer (NK) cells.

## 2. Materials and methods

### 2.1. Cells and cell culture

All cells were periodically screened (Gen-probe, H. Biermann, FRG) and defined as negative for *mycoplasma* contaminations.

The human 5838 Ewing's Sarcoma (ES) cell line as described by R. B. Herberman and colleagues (McCoy *et al.* 1977), was kindly provided by Dr M. L. Meltz (University of Texas, San Antonio, USA). Exponentially growing ES cells were cultured in 25 cm<sup>2</sup> flasks (Nunc, Denmark) as monolayer cell lines in a standard RPMI 1640 medium supplemented with 10% FCS, 6 mM L-glutamine and antibiotics; the cells were reseeded and splitted every four days. The doubling time for ES cells at 37°C was 24 h and the plating efficiency (PE) ranged from 70 to 80%.

Leukemic K562 cells were cultured in the same RPMI 1640 medium as indicated above.

Peripheral blood obtained from healthy human volunteers was anti-coagulated with heparin (Heparin Novo, Mainz, FRG). Peripheral blood mononuclear cells (PBMC) were separated on Ficoll Isopaque (Ficoll Paque, Pharmacia, Sweden) density gradient centrifugation. After separation peripheral blood lymphocytes (PBL) were obtained and washed 2x in RPMI 1640 (Gibco, UK) supplemented with 10% heat inactivated foetal calf serum (FCS, Gibco, UK), 6 mM L-glutamin (Gibco, UK).

Human Epstein-Barr virus (EBV) transformed B-lymphoblastoid cellines (B-LCL) were established in our laboratory from freshly isolated PBL. Briefly, PBL were



incubated in 5 ml RPMI 1640 medium containing 15% FCS, 6 mM L-glutamine, antibiotics and equal volume of EBV containing supernatant (B98-8, ATCC) for 10–14 days. Phytohaemagglutinin (PHA-M, Wellcome Diagnostics, UK) was added to a final concentration of 1%. After about three weeks permanently growing EBV transformed B-LCL were generated.

NK enriched effector cells were generated by plastic adherence and by stimulation with recombinant IL-2 (1001 U.) according to a method described previously (Multhoff *et al.* 1995b).

## 2.2. Treatment

Exponentially growing cells were treated at the non-lethal temperature of 41.8°C, for 2 h in a temperature-controlled waterbath (Haake E3, Karlsruhe, FRG). After a recovery period of 12 h at 37°C the heat stressed and untreated cells were used for indirect immunofluorescence studies and for cytotoxicity assays.

Inhibition of RNA- and protein-synthesis were performed using either actinomycin D (AcD) or cycloheximide (CHX) on untreated (37°C) or non-lethally heat treated (41.8°C 2 h) cells. The cells were incubated with 2 µg actinomycin D (A1410, Sigma, USA) or 10 µg cycloheximide (C-6679, Sigma, USA) per  $1 \times 10^6$  cells for either 6 h (prior to heat treatment) or directly after heat treatment. After the incubation period the cells were washed twice with PBS/10% FCS and used for indirect immunofluorescence studies followed by FACScan analysis.

## 2.3. Indirect immunofluorescence analysis and flowcytometry

Cell surface expression of HSP72 and MHC class I molecules was measured before and after treatment with cycloheximide using indirect immunofluorescence analysis. Viable cells, either untreated or heat treated  $0.5 \times 10^6$  each, were trypsinized and washed 2× with PBS/10% FCS. Cells were then incubated for 1 h at 4°C with the following monoclonal antibodies (moAb) each containing 0.1% sodium azide ( $\text{NaN}_3$ ). The isotype of each moAb is given in parenthesis: anti-HSP72 (IgG1, Amersham, USA; Welch and Feramisco 1984), W6/32 (IgG2a, anti MHC class I, ATCC), IgG1 and IgG2a isotype matched antibodies as negative controls. Washed cell pellets were incubated with fluorescein-isothiocyanate-conjugated rabbit anti-mouse IgG (FITC, Dako, Hamburg, FRG) at 4°C for 30 min.

Quantitative flowcytometric analysis were done using a fluorescence-activated cell sorter (FACScan instrument Becton Dickinson, UK). The percentage positively stained cells was calculated by the difference of the number of specifically stained cells minus the number of cells stained with isotype-matched control antibodies. For each sample 3000 to 10000 viable cells were analysed. Viability of the cells was demonstrated by negative propidium-iodide staining and by trypan-blue exclusion.

## 2.4. Cytotoxicity assays (CMT)

The specificity of IL-2 stimulated NK enriched effector cells was monitored in a standard Cr-51 release assay as described elsewhere (Multhoff *et al.* 1995b). Ewing's Sarcoma cells, leukemic K562 cells and EBV transformed allogeneic B-LCL, either untreated or heat treated at non-lethal temperatures (41.8°C for 2 h followed by a recovery period at 37°C for 12 h), were used as target cells. Percent specific lysis was

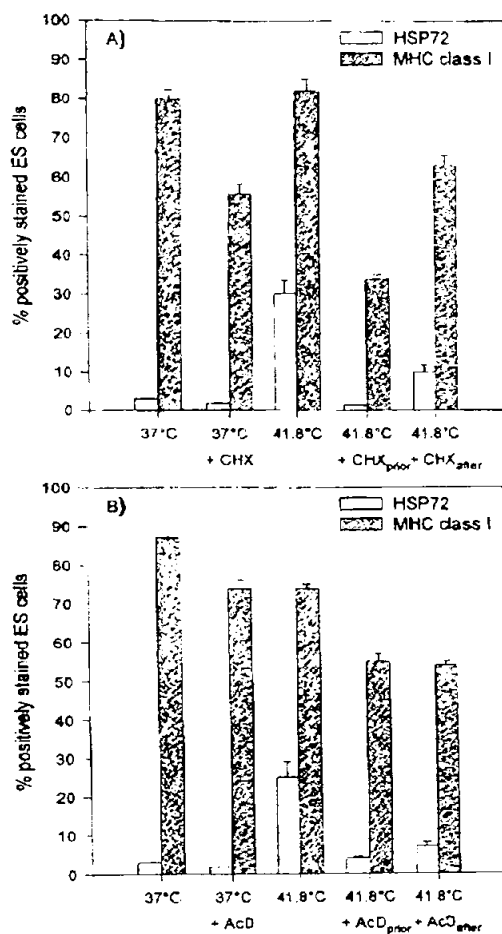


Figure 1. (A) Effect of cycloheximide (CHX;  $10 \mu\text{g}/1 \times 10^6$  cells) on cell surface expression of HSP72 (white bars) and MHC class I molecules (hatched bars) on either untreated ( $37^\circ\text{C}$ ) or heat shocked ( $41.8^\circ\text{C}$  2 h, followed by a recovery period at  $37^\circ\text{C}$  for 4 h) ES cells.  $37^\circ\text{C}$ : untreated ES cells;  $37^\circ\text{C} + \text{CHX}$ : untreated ES cells with cycloheximide;  $41.8^\circ\text{C}$ : heat shocked ES cells;  $41.8^\circ\text{C} + \text{CHX}_{\text{prior}}$ : cycloheximide prior to heat shock of ES cells;  $41.8^\circ\text{C} + \text{CHX}_{\text{after}}$ : cycloheximide after heat shock of ES cells. Cell viability was over 95% in all cases; the bars represent the SD values of 3 independent experiments. Similar results were obtained with actinomycin D (AcD). (B) Effect of actinomycin D (AcD;  $2 \mu\text{g}/1 \times 10^6$  cells) on cell surface expression of HSP72 (white bars) and MHC class I molecules (hatched bars) on either untreated ( $37^\circ\text{C}$ ) or heat shocked ( $41.8^\circ\text{C}$  2 h, followed by a recovery period at  $37^\circ\text{C}$  for 12 h) ES cells.  $37^\circ\text{C}$ : untreated ES cells;  $37^\circ\text{C} + \text{AcD}$ : untreated ES cells with cycloheximide;  $41.8^\circ\text{C}$ : heat shocked ES cells;  $41.8^\circ\text{C} + \text{AcD}_{\text{prior}}$ : cycloheximide prior to heat shock of ES cells;  $41.8^\circ\text{C} + \text{AcD}_{\text{after}}$ : cycloheximide after heat shock of ES cells.

Cold target inhibition CML assays were performed by using heat treated K562, ES and B-LCL as unlabelled target cells at a 'cold' to 'hot' target cell ratio ranging from 20:1 to 2:1. The effector to target ratio in these experiments was constant at 20:1.

**2.1. Results**

In a clinical phase II study patients with deep seated sarcomas of the abdomen and pelvis received regional hyperthermia in combination with chemotherapy (Issels *et al.* 1990). Our aim was to analyse the immunological aspects of a non-lethal heat treatment on normal and diseased cells. For our studies we used a human malignant Ewing's Sarcoma cell line (ES) derived from a tumour patient (kindly provided by Dr M. L. Meltz, University of San Antonio, USA), leukemic K562 cells and EBV transformed B-LCL derived from healthy human individuals. The non-lethal heating conditions for all three cell lines were defined in a clonogenic cell survival assay. As previously demonstrated (Multhoff *et al.* 1995a), a temperature of 41.8°C was defined as non-lethal for all three cell lines. In order to keep cells alive for further immunological investigations a temperature of 41.8°C and an incubation period of 1 h was used.

With regard to our previous findings that HSP72 is selectively expressed on the cell surface of tumour (ES, K562) cells but not on normal cells (B-LCL) after heat shock (Multhoff *et al.* 1995a), we addressed the question whether heat-induced HSP72 cell surface expression is dependent on *de novo* protein synthesis. In order to answer this question indirect immunofluorescence studies followed by quantitative flowcytometry using the HSP72 and MHC class I specific moAbs were performed using either untreated (37°C) or heat treated (41-8°C 2 h; followed by a recovery period at 37°C for 4 h) cells that were incubated with cycloheximide (CHX) or actinomycin D (AcD) either directly before heat shock or after heat shock. The results obtained from this protein- and RNA-synthesis inhibition study is shown in Figure 1 A, B. The cell surface expression pattern of HSP72 is indicated by white bars and of MHC class I expression by hatched bars. Under physiological conditions an incubation with CHX or with AcD had no significant effect on the cell surface expression pattern of HSP72 and MHC class I molecules. As previously shown (Multhoff *et al.* 1995a), HSP72 cell surface expression could be induced by non-lethal heat shock on about 30% of the sarcoma cells. Under these heating conditions the MHC class I expression was not influenced. However, an inhibition of protein- or RNA-synthesis with cycloheximide or actinomycin D prior to non-lethal heat treatment, results in a total inhibition of cell surface expression of HSP72. A weaker inhibition in the cell surface expression pattern of HSP72 and MHC class I molecules was observed if the protein synthesis inhibitors were added after heat shock. Similar results were obtained with leukemic K562 cells; no effect of CHX was seen with B-LCL that lack to express HSP72 on the cell surface (data not shown).

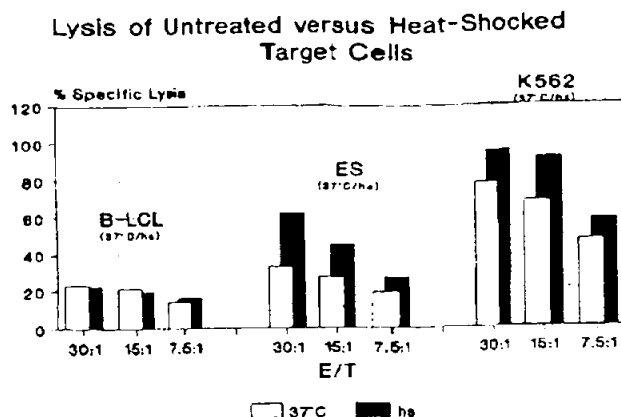


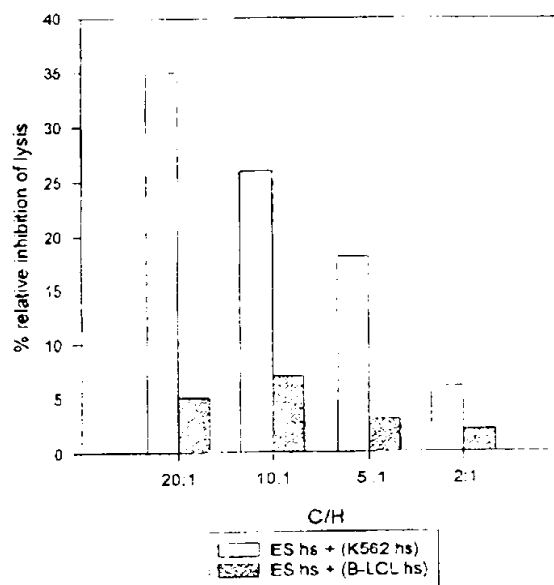
Figure 2. Comparison of the lysis of untreated (37°C) and heat shocked (41-48°C 2 h, followed by a recovery period at 37°C for 4 h) B-LCL, ES and K562 cells. E/T ratios ranging from 30:1 to 7.5:1; % spontaneous release of each target cell was always < 20%. The data represent the mean value of 4 independent experiments; SD values for each data point was < 10%.

### 3.2. Increased sensitivity to lysis against NK enriched effector cells could be blocked by HSP72 specific mAb

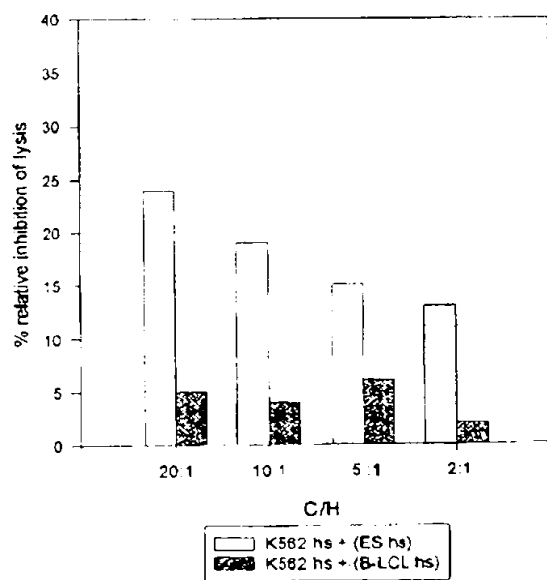
NK enriched, IL-2 stimulated cytotoxic effector cells were used to compare the lysis of untreated and heat treated tumour (ES, K562) and normal cells. As shown in Figure 2, there was nearly no lysis of allogeneic B-LCL at E/T ratios ranging from 30:1 to 7.5:1. Even after heat shock there was no increase in the lysis of B-LCL that lack to express HSP72 on the cell surface (Multhoff *et al.* 1995a). The lysis of leukemic K562 cells was higher compared to ES cells under physiological conditions. However, after non-lethal heat shock there was a significant increase in the sensitivity to lysis of both tumour cell types. This result could be correlated with an increase in cell surface expression of HSP72. These data could be confirmed by antibody blocking studies: the increased lysis of ES and K562 cells after heat shock could be inhibited by an HSP72 specific antibody but not by an MHC class I specific or an isotype control antibody. No inhibition of lysis with the HSP72 specific antibody was observed with untreated and heat treated B-LCL (Multhoff *et al.* 1995b).

Figure 3. (A) Cold target inhibition assay: heat treated K562 cells (K562 hs) or B-LCL (B-LCL hs) were used as 'cold' target cells for the inhibition of lysis of 'hot' heat treated ES cells. No inhibitory effect on the lysis of heat treated ES cells was observed with 'cold' heat treated B-LCL (B-LCL hs). However, 'cold' heat treated K562 cells (K562 hs) were able to inhibit the lysis of heat shocked ES cells in a concentration dependent manner. (B) Cold target inhibition assay: heat treated ES cells (ES hs) or B-LCL (B-LCL hs) were used for the inhibition of lysis of 'hot' heat treated K562 cells. No inhibitory effect on the lysis of heat treated K562 cells was observed with 'cold' heat treated B-LCL (B-LCL hs). However, 'cold' heat treated ES cells (ES hs) were able to inhibit the lysis of heat shocked K562 cells in a concentration dependent manner. The relative rate of inhibition (%) is plotted as a function of chromium release without cold target cells; % spontaneous release in both assays was always < 15%. The data represent the mean values of 3 independent experiments; SD values for each data point was < 10%.

A) Cold Target Inhibition



B) Cold Target Inhibition



### 3.3. Cold target inhibition of different HSP72 expressing tumour cell types

A cold target inhibition experiment was performed using heat shocked Ewing's Sarcoma (ES) and leukemic K562 cells as unlabelled, 'cold' target cells in order to figure out the role of HSP72 as an immunogenic determinant on different tumour cell types. Heat shocked B-LCL derived from healthy human donors, that lack to express HSP72 on their cell surface were used as a control. As shown in Figure 3A the lysis of heat shocked ES could not be inhibited by adding heat shocked B-LCL. However, a strong inhibition of lysis of heat shocked ES cells was observed by using K562 cells that were exposed to a non-lethal heat dose (Figure 3A) as 'cold' target cells. Similar results were obtained in the reversed situation when heat shocked ES cells and B-LCL were used as 'cold' target cells for inhibition of lysis of heat treated K562 cells (Figure 3B).

## 4. Discussion

Traditionally, HSP are known to be located intracellularly where they perform a variety of chaperoning functions (Welch 1992). Recently, evidence is accumulating that especially members of the HSP70 (Konno *et al.* 1989, Ferrarini *et al.* 1992, Heufelder *et al.* 1992) families are also expressed on the cell surface.

Cellular stress, such as non-lethal heat, results in a comparable strong induction of HSP72 in normal and diseased cell types that results in a strong increase in the cytoplasmic amount of HSP72 (Multhoff *et al.* 1995a). However, non-lethal heat treatment followed by a recovery period at 37°C induces cell surface expression of HSP72 selectively on human sarcoma and leukemic cells, but not on normal cells that was detected by immunological and biochemical methods. For immunologists the interesting aspects of HSP cell surface expression are the consequences for inducing a specific anti-tumour immune response (DeNagel and Pierce 1993, Li and Srivastava 1993). We could demonstrate that HSP72 could act as a heat-inducible immunogenic target structure on human sarcoma and leukemic cells that is recognized by non-MHC restricted NK effector cells (Multhoff *et al.* 1995b). However, little is known about the cellular pathways used by HSP from the cytosol to the plasma membrane. Most HSP (with the exception of gp96) do not possess signal peptides (Booth and Koch 1989), nevertheless they are present in the Golgi, ER, nuclei, nucleoli and on the cell surface, indicating that HSP are transported by indirect means. Hightower and Guidon (1989) suggested that rapidly released proteins, such as HSP, are not transported via common secretory pathways. Inhibitors of the ER-Golgi secretory pathway do not block their release (Multhoff and Hightower 1996) thus indicating that HSP use a yet undefined alternative transport pathway. In order to clarify the question whether *de novo* protein synthesis is necessary for the cell surface expression of HSP72 on human tumour cells, experiments were performed using either the protein synthesis inhibitor cycloheximide or the RNA synthesis inhibitor actinomycin D. The data clearly indicate that the heat-induced cell surface expression of HSP72 is dependent on protein synthesis. Furthermore, we wanted to know whether sarcoma and leukemic cells differ in their HSP72 immunogenic determinant that is recognized by non-MHC restricted NK cells. Cold target inhibition assays revealed that heat shocked sarcoma and leukemic cells have to express very similar if not identical HSP72 epitopes that compete with each other, since the lysis of heat shocked sarcoma cells could be inhibited by the addition of unlabelled but heat shocked leukemic cells and vice versa.

In summary we can state that histologically different, sensitive human tumour cell types express a very similar if not identical immunogenic HSP72 determinant after non-lethal hyperthermia treatment that acts as a tumour-specific recognition structure for NK cells.

#### Acknowledgements

This work was supported in part by Grant M 90/91/Is1 from the Deutsche Krebshilfe and by Grant Is 31/3-2 from the Deutsche Forschungsgemeinschaft.

#### References

- BIENZ M., and PELHAM H. R., 1987, Mechanisms of heat-shock gene activation in higher eukaryotes. *Advances in Genetics*, **24**, 31-72.
- BOOTH C., and KOCH G. L. E., 1989, Perturbation of cellular calcium induces secretion of luminal ER proteins. *Cell*, **59**, 729-737.
- BOTZLER C., KOLB H. J., ISSELS R. D., and MÜLTHOFF G., 1996, Nontoxic alkyl-lysophospholipid treatment increases sensitivity of leukemic K562 cells to lysis by natural killer cells. *International Journal of Cancer*, **65**, 633-638.
- CHIRICO W. J., WATERS M. G., and BLOBEL G., 1988, 70K heat shock related proteins stimulate protein translocation into microsomes. *Nature*, **332**, 805-810.
- DEHAGEL D. C., and PIERCE S. K., 1993, Heat shock proteins in immune responses. *Critical Reviews in Immunology*, **13**, 71-81.
- DICESAPE S., POCCIA F., MASTINO A., and COLIZZI V., 1992, Surface expressed heat-shock proteins by stressed or human immunodeficiency virus (HIV)-infected lymphoid cells represent the target for antibody-dependent cellular cytotoxicity. *Immunology*, **76**, 341-343.
- FERRARINI M., HELTAI S., ZOCCHI M. R., and RUGARLI C., 1992, Unusual expression and localization of heat-shock proteins in human cells. *International Journal of Cancer*, **51**, 613-619.
- HEUFELDER A. E., WENZEL B. E., and BAHN R. S., 1992, Cell surface localization of a 72 kDa heat shock protein in retroocular fibroblasts from patients with Graves' ophthalmopathy. *Journal of Clinical Endocrinology and Metabolism*, **74**, 732-736.
- HIGHTOWER L. E., and GUIDON P. T., 1989, Selective release from cultured mammalian cells of heat-shock (stress) proteins that resemble glia-axon transfer proteins. *Journal of Cell Physiology*, **138**, 257-266.
- ISSELS R. D., PRENNINGER S. W., NAGELE A., BOEHM E., SAUER H., JAUCH K. W., DENECKE H., BERGER H., PETER K., and WILMANN W., 1990, Ifosfamide plus etoposide combined with regional hyperthermia in patients with locally advanced sarcomas: a phase II study. *Journal of Clinical Oncology*, **8**, 1818-1829.
- KONNO A., SAITO N., YAGIHASHI A., TORIGOE T., CHO J. M., TORIMOTO K., HARA I., WADA Y., OKUBO M., and TAKAHASHI N., 1989, Heat- or stress-inducible transformation-associated cell surface antigen on the activated H-ras oncogene-transfected rat fibroblast. *Cancer Research*, **49**, 6578-6582.
- LI Z., and SRIVASTAVA P. K., 1993, Tumor rejection antigen gp96/grp97 is an ATPase: implications for folding and antigen presentation. *EMBO Journal*, **12**, 3343-3351.
- LINDQUIST S., 1986, The heat-shock response. *Annu. Rev. Biochem.*, **55**, 1151-1191.
- MCCOY J. L., JEROME L. J., CANNON G. B., POMEROY T. C., CONNOR R. J., OLDHAM R. K., WEESE J. L., and HERBERMANN R. B., 1977, Leukocyte migration inhibition in patients with Ewing's sarcoma by 3-M potassium chloride extracts of fresh and tissue-cultured Ewing's sarcoma. *Journal of the National Cancer Institute*, **59**, 1119-1125.
- MORIMOTO R. I., 1993, Cells in stress: transcriptional activation of heat shock genes. *Science*, **259**, 1409-1410.
- MÜLTHOFF G., BOTZLER C., WIESNER M., MÜLLER E., MEIER T., and ISSELS R. D., 1995a, A stress-inducible 72 kDa heat shock protein (HSP72) is expressed on the surface of human tumor cells, but not on normal cells. *International Journal of Cancer*, **61**, 272-279.
- MÜLTHOFF G., BOTZLER C., WIESNER M., EIGNER G., and ISSELS R., 1995b, CD3<sup>+</sup> large granular lymphocytes recognize a heat-inducible immunogenic determinant

- associated with the 72-kDa heat shock protein on human sarcoma cells. *Blood*, **86**, 1374-1382.
- MULTHOFF, G., and HIGHTOWER, L., 1996, Cell surface expression of HSP and the immune response. *Cell Stress and Chaperones*, **1**, 3.
- PELLIAM, H. R., 1986, Speculations on the functions of the major heat shock and glucocorticoid-regulated proteins. *Cell*, **46**, 959-961.
- SATO, N., CHO, J. M., TAKASHIMA, T., TSUBOI, N., NIHEI, T., WADA, Y., and KIKUCHI, K., 1991, Immunogenicity and involvement in the host's effector mechanisms of the oncogene-induced transformation-associated cell surface antigens. *In Vivo*, **5**, 663-668.
- SCHLESINGER, M. J., ASHURNER, M., and TISSIERES, A. (eds), 1982, Heat shock: from bacteria to man. *Cold Spring Harbor 1982* (New York: Cold Spring Harbor Laboratory Press), pp. 1-297.
- SRIVASTAVA, P. K., DELEO, A. B., and OLD, L. J., 1986, Tumor rejection antigens of chemically induced sarcomas of inbred mice. *Proceedings of the National Academy of Science USA*, **83**, 3407-3411.
- TAMURA, Y., TSUBOI, N., SATO, N., and KIKUCHI, K., 1993, 70 kDa heat shock cognate protein is a transformation-associated antigen and a possible target for the host's anti-tumor immunity. *Journal of Immunology*, **151**, 5516-5524.
- ULLRICH, S. J., ROBINSON, E. A., LAW, L. W., WILLINGHAM, M., and APPELLA, E. A., 1986, Mouse tumor-specific transplantation antigen is a heat shock-related protein. *Proceedings of the National Academy of Science, USA*, **83**, 3121-3125.
- VANBUSKIRK, A., CRUMP, B. J., MARGOLASH, E., and PIERCE, S. K., 1989, A peptide binding protein having a role in antigen presentation is a member of the HSP70 heat shock family. *Journal of Experimental Medicine*, **170**, 1799-1809.
- WELCH, W. J., 1992, Mammalian stress response: cell physiology, structure/function of stress proteins, and implications for medicine and disease. *Physiological Review*, **72**, 1063-1081.
- WELCH, W. J., and FERAMISCO, J. R., 1984, Nuclear and nucleolar localization of the 72,000 Dalton heat shock protein in heat shocked mammalian cells. *Journal of Biological Chemistry*, **259**, 4501-4513.



## PATENT COOPERATION TREATY

PCT

## NOTIFICATION OF ELECTION

(PCT Rule 61.2)

From the INTERNATIONAL BUREAU

To:

Commissioner  
 US Department of Commerce  
 United States Patent and Trademark  
 Office, PCT  
 2011 South Clark Place Room  
 CP2-5C24  
 Arlington, VA 22202  
 ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

<b>Date of mailing</b> (day month year) 18 June 2001 (18.06.01)	
<b>International application No.</b> PCT/GB00/03225	<b>Applicant's or agent's file reference</b> P2661PC/TIPD
<b>International filing date</b> (day month year) 18 August 2000 (18.08.00)	<b>Priority date</b> (day month year) 19 August 1999 (19.08.99)
<b>Applicant</b> COLACO, Camilo, Anthony, Leo, Selwyn	

1. The designated Office is hereby notified of its election made:

☒ in the demand filed with the International Preliminary Examining Authority on:

13 March 2001 (13.03.01)

☐ in a notice effecting later election filed with the International Bureau on:2. The election ☒ was☐ was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO  
 34, chemin des Colombettes  
 1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer

Olivia TEFY

Telephone No.: (41-22) 338.83.35

\_\_\_\_\_

## PATENT COOPERATION TREATY

PCT

From the INTERNATIONAL BUREAU

NOTIFICATION OF THE RECORDING  
OF A CHANGE(PCT Rule 92bis.1 and  
Administrative Instructions, Section 422)

To:

MURGITROYD & COMPANY  
Chartered Patent Agents  
373 Scotland Street  
Glasgow G5 8QA  
ROYAUME-UNI

Date of mailing (day month year) 15 October 2001 (15.10.01)	IMPORTANT NOTIFICATION
Applicant's or agent's file reference P2661PC/TIPD	
International application No. PCT/GB00/03225	International filing date (day month year) 18 August 2000 (18.08.00)

1. The following indications appeared on record concerning:		
<input type="checkbox"/> the applicant	<input type="checkbox"/> the inventor	<input checked="" type="checkbox"/> the agent
<input type="checkbox"/> the common representative		
Name and Address DUMMETT, Thomas, Ian, Peter Dummett Copp 25 The Square Martlesham Heath Ipswich IP5 3SL United Kingdom	State of Nationality	State of Residence
	Telephone No. 01473 660600	
	Facsimile No. 01473 660612	
	Teleprinter No.	
2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:		
<input checked="" type="checkbox"/> the person	<input checked="" type="checkbox"/> the name	<input checked="" type="checkbox"/> the address
<input type="checkbox"/> the nationality		
<input type="checkbox"/> the residence		
Name and Address MURGITROYD & COMPANY Chartered Patent Agents 373 Scotland Street Glasgow G5 8QA United Kingdom	State of Nationality	State of Residence
	Telephone No. 141 307 8400	
	Facsimile No. 141 307 8401	
	Teleprinter No.	
3. Further observations, if necessary:		
4. A copy of this notification has been sent to:		
<input checked="" type="checkbox"/> the receiving Office	<input type="checkbox"/> the designated Offices concerned	
<input type="checkbox"/> the International Searching Authority	<input checked="" type="checkbox"/> the elected Offices concerned	
<input checked="" type="checkbox"/> the International Preliminary Examining Authority	<input type="checkbox"/> other:	

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland	Authorized officer S. Buttay
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

The first part of the paper discusses the importance of the research and the objectives of the study. It then presents a literature review of the existing research on the topic. The methodology section describes the research design and the data collection process. The results section presents the findings of the study, and the conclusion section summarizes the main findings and provides recommendations for future research.

The study was conducted in a laboratory setting, and the data were collected using a series of experiments. The results of the experiments were analyzed using statistical methods, and the findings were compared with the results of previous studies. The study found that the research objectives were achieved, and the results were consistent with the findings of previous research.

The study has several limitations, and there are some areas that need to be explored in future research. The study was conducted in a laboratory setting, and the results may not be generalizable to real-world situations. The study also had a limited sample size, and the results may be affected by the characteristics of the sample.

In conclusion, the study provides valuable insights into the research topic, and the findings are consistent with the existing literature. The study also identifies some areas for future research, and the results provide a basis for further exploration of the topic.

# PATENT COOPERATION TREATY

PCT

## NOTIFICATION CONCERNING SUBMISSION OR TRANSMITTAL OF PRIORITY DOCUMENT

(PCT Administrative Instructions, Section 411)

From the INTERNATIONAL BUREAU

To:

MURGITROYD & COMPANY  
Chartered Patent Agents  
373 Scotland Street  
Glasgow G5 8QA  
ROYAUME-UNI

Date of mailing (day month year)

14 June 2002 (14.06.02)

Applicant's or agent's file reference

P2661PC/TIPD

### IMPORTANT NOTIFICATION

International application No.

PCT/GB00/03225

International filing date (day month year)

18 August 2000 (18.08.00)

International publication date (day month year)

01 March 2001 (01.03.01)

Priority date (day month year)

19 August 1999 (19.08.99)

Applicant

IMMUNOBIOLOGY LIMITED et al

1. The applicant is hereby notified of the date of receipt (except where the letters "NR" appear in the right-hand column) by the International Bureau of the priority document(s) relating to the earlier application(s) indicated below. Unless otherwise indicated by an asterisk appearing next to a date of receipt, or by the letters "NR", in the right-hand column, the priority document concerned was submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b).
2. This updates and replaces any previously issued notification concerning submission or transmittal of priority documents.
3. An **asterisk(\*)** appearing next to a date of receipt, in the right-hand column, denotes a priority document submitted or transmitted to the International Bureau but not in compliance with Rule 17.1(a) or (b). In such a case, **the attention of the applicant is directed** to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.
4. The letters "NR" appearing in the right-hand column denote a priority document which was not received by the International Bureau or which the applicant did not request the receiving Office to prepare and transmit to the International Bureau, as provided by Rule 17.1(a) or (b), respectively. In such a case, **the attention of the applicant is directed** to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

Priority date
Priority application No.
Country or regional Office  
or PCT receiving Office
Date of receipt  
of priority document

19 Augu 1999 (19.08.99)

9919733.7

GB

08 May 2002 (08.05.02)

The International Bureau of WIPO  
34, chemin des Colombettes  
1211 Geneva 20, Switzerland

Authorized officer

PETRESKA Gorica

Facsimile No. +41 22 338 83 35

Telephone No. +41 22 338 83 38

